UCRL-11731

University of California

Ernest O. Lawrence Radiation Laboratory

ABSORPTION CHANGES IN BACTERIAL CHROMATOPHORES

II. A NEW CHLOROPHYLL-LIKE PIGMENT FROM THE OXIDATION OF CHROMATOPHORES FROM RHODOSPIRILLUM RUBRUM

Berkeley, California

Rept. submitted for publication in
Photochemistry and Photobiology.

UCRL-11731

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

AEC Contract No. W-7405-eng-48

ABSORPTION CHANGES IN BACTERIAL CHROMATOPHORES

II. A NEW CHLOROPHYLL-LIKE PIGMENT FROM THE OXIDATION OF CHROMATOPHORES FROM RHODOSPIRILLUM RUBRUM

Edwin S. Gould, Irwin D. Kuntz, Jr., and Melvin Calvin

October, 1964

ABSORPTION CHANGES IN BACTERIAL CHROMATOPHORES. II. A NEW CHLOROPHYLL-LIKE PIGMENT FROM THE OXIDATION OF CHROMATOPHORES FROM RHODOSPIRILLUM RUBRUM

Edwin S. Gould,* Irwin D. Kuntz, Jr.,** and Melvin Calvin

Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory,*** and Department of Chemistry, University of California, Berkeley, California

Abstract--Evidence is presented which points to (at least) two bound forms of bacteriochlorophyll present in chromatophores of Rhodospirillum rubrum, both of them readily converted to unbound bacteriochlorophyll (abs. max. 770 mμ) when the chromatophores are extracted with acetone or ethanol. Controlled oxidation of the chromatophores with Ir(IV) or with Zn(II) and ferricyanide preferentially destroys the more strongly absorbing pigment (abs. max. 880 mμ) but brings about only a slight decrease in the magnitude of the photoinduced absorption changes at 810 and 792 mμ. Such oxidations yield a new pigment, absorbing at 715 mμ in the aqueous preparation and, more strongly, at 680-684 mμ when the pigment is extracted into organic solvents. This pigment is formed irreversibly and is therefore different from the material formed by photooxidation of chromatophores. Its visible spectrum and the spectrum of the material formed from it by acidification suggest that it is a chlorophyll-like substance, possibly derived from bacteriochlorophyll by (two-electron) oxidation of one of the dihydropyrrole rings to a pyrrole ring. Directions are given for separation of this pigment from other colored compounds present in the oxidation mixtures.

*National Institutes of Health Special Postdoctoral Fellow, University of California, 1963-64. Present address: Stanford Research Institute, Menlo Park, California.

**National Science Foundation Fellow, 1963-64.

***This work was sponsored, in part, by the U. S. Atomic Energy Commission.

There is a growing body of evidence which suggests that photoinduced absorption changes in bacterial systems are related to photosynthesis. In certain bacterial chromatophores, such changes are accompanied by the production of unpaired electrons (9), and may be reversibly duplicated by careful treatment with such "one-electron" oxidants as hexachloroiridate(IV) or ferricyanide. (8) An interesting facet of such oxidations is the irreversible bleaching of the principal pigment which often occurs.

For example, oxidation of chromatophores from R. rubrum with $IrCl_4^=$ or $Fe(CN)_6^{3-}$ has been shown to remove much of the absorbance at 880 mμ with, however, little or no decrease in the magnitude of the photoinduced absorption changes (8) or the ESR signal. (9) When chromatophore preparations are extracted with ethanol or acetone at various stages during the progress of such oxidation, the height of the bacteriochlorophyll peak at 770 mμ (5) (in the organic solvent) decreases as the oxidation proceeds. When the oxidation is continued until the peak at 880 mμ disappears, leaving a much smaller peak at 810 mμ, extracts of the resulting preparation still exhibit an absorption peak at 770 mμ with a height corresponding to about 20% of the bacteriochlorophyll present in the unoxidized chromatophores, in keeping with the view (9, 8, 2) that the bacteriochlorophyll in such chromatophores is present in (at least) two forms which differ markedly

in their photochemical activity and in the ease with which they are oxidized.

During such oxidations, the disappearance of the 880 mμ peak is accompanied by the appearance of a much smaller peak (in water) at 715 mμ which, upon extraction into acetone, ethanol, or ether, leads to a relatively more intense peak at 680-684 mμ.

The pigment exhibiting this absorbance is not present in significant amount in unoxidized chromatophores. Its concentration increases during the early stages of oxidation, but with excess oxidant, it is slowly consumed. The conversion appears to be irreversible, for treatment of the new pigment, either in aqueous or in organic media, with ferrocyanide or with dithionite fails to restore the bacteriochlorophyll absorbance. The pigment thus does not correspond to the material which is reversibly formed by photooxidation or careful chemical oxidation of rubrum chromatophores. Moreover, it presumably does not itself undergo photooxidation, for the photoinduced spectrum of chromatophores which have been partially oxidized with Ir(IV) shows no dip at 715 mμ comparable to the very large minima at 865-870 mμ.

The spectrum of the (grass-green) pigment, which may be separated from residual non-oxidized bacteriochlorophyll and from the carotenoids present in chromatophores by chromatography on powdered polyethylene (1), is shown in Figure 1, together with the spectrum resulting from acidification of the solution with HCl.

The positions of the two principal maxima of the pigment, together with the character of the spectral shifts upon acidification, indicate strongly that the green pigment is a chlorophyll-like material and that

acidification has produced a pheophytin. (11) No isolation of a chlorophyll having spectral characteristics corresponding to our pigment appears to have yet been reported, although there is some evidence that this pigment is formed by the oxidation of R. rubrum with benzoquinone. (7) Concentration of solutions of the pigment yielded a black gum, but the pigment has not yet been obtained in crystalline form.

The similarity of its visible and ultraviolet spectrum to that of chlorophyll d (11, 10) and its mode of formation by mild oxidation of bacteriochlorophyll suggests that our pigment is derived from bacteriochlorophyll by oxidation of one (but not both) of the dihydropyrrole rings to a pyrrole ring. (6)

EXPERIMENTAL

Changes in photoinduced absorption changes with oxidation. Both the preparation of chromatophores from Rhodospirillum rubrum (8) and the method for measuring photoinduced absorption changes (9) have been described. Oxidations with Ir(IV) were carried out by adding small portions of a solution of K_2IrCL_6 (10 mg/ml) to a suspension of chromatophores buffered at pH 7 (0.01 F Na_2HPO_4 + 0.01 F NaH_2PO_4); such absorption changes that occurred took place almost immediately at 0° C. For the ferricyanide oxidations, solutions were 0.2 F in $K_3Fe(CN)_6$ and 0.05 F in $ZnSO_4$; here, absorption changes were much slower and occurred over a period of 3-8 hours, even at 25° C. Our chromatophore suspensions were less resistant to oxidation than those reported previously; (9) typically, 6 ml of chromatophore suspension, optical density 3.0 at 880 mμ (containing about 6 mg of dried chromatophores) required less than 3 mg of K_2IrCl_6 at 0° for oxidation to the stage at which absorptions at 820 and 715 mμ were comparable.

In our hands, there was slight but definite diminution in the magnitude of the photoinduced absorption during the early stages of oxidation, both with ferricyanide and with Ir(IV). Figure 2 indicates the progress of the relative light-minus-dark response, taken as the difference in absorbance between the minimum at 810 mμ and the maximum at 792 mμ in the photoinduced spectrum, as the pigment(s) absorbing near 880 mμ is consumed by oxidation. Typically, only about 10% of the photoinduced absorption changes are affected by oxidizing away about 70% of the peak near 880, and almost 40% of the light-minus-dark response remains, even when the (shifted) peak height near 810 mμ has become less than 10% of the 880 peak in the unoxidized chromatophores.

There is some indication that in the presence of the surfactant Triton X-100 (0.5% aqueous solution), the oxidation with Zn(II) and ferricyanide may be even more selective. In two separate experiments with this combination of reagents, about 90% of the light-dark response remained after the absorption peak (in this case, near 860 mμ) had dropped to 15-17% of its height in the unoxidized material. However, the procedure is capricious; often the oxidation was extremely sluggish and much less of the photoinduced response was observed with the partially oxidized preparation. Oxidations of chromatophores with cerium(IV) sulfate and the o-phenanthroline complex of iron(III) were not promising; with both of these reagents, rapid coagulation and precipitation occurred. Oxidations with ferricyanide (in the absence of zinc and triton), with hydrogen peroxide, and with air were inconveniently slow at room temperatures.

Absorbance of bacteriochlorophyll in acetone extracts of partially oxidized chromatophores. In a number of experiments, the fall-off of the 880 peak during the oxidation of chromatophores was compared with the diminution of the bacteriochlorophyll peak at 770 m in the acetone extracts of the partially oxidized material. Since bacteriochlorophyll solutions in organic solvents are readily oxidized by air, extraction of chromatophores and spectral measurements were carried out under nitrogen. Typically, a sample (original optical density about 3.0 at 880 mμ) partially oxidized with Ir(IV) was measured into a 1 cm cuvette, two drops of 1 F sodium dithionite added, the cuvette tightly covered with a rubber serum cap, and air removed by flushing with nitrogen. The sample was then diluted with from 10 to 30 times its volume of acetone (which had previously been boiled to remove dissolved air), adding the diluent through the cap using a volumetric syringe. When the precipitated protein caused difficulty during spectral measurements, the capped cuvette was wrapped in foam rubber to protect the edges and corners, placed in a 40 ml centrifuge tube, and centrifuged.

In Figure 3, the absorbancies at 770 mμ (relative to the absorbance in an extract of unoxidized chromatophores) are compared to the absorbancies of the chromatophore preparations from which the extracts are derived. In Curve A, the chromatophore absorbancies at 880 mμ are plotted; Curve B represents the relative absorbancies at the peak near 880. At first the two curves coincide, but as the chromatophore peak position shifts to shorter wavelengths, the optical density at 880 falls well below that at the peak positions. During the early stages of the oxidation, the 880 peak height for the chromatophores falls rapidly and the bacteriochlorophyll

peak at 770 in the acetone extract disappears much more slowly as the most strongly absorbing pigment in the chromatophores is preferentially consumed. Thus, when the 880 absorbance has dropped about 75%, the 770 absorbance in acetone has dropped only about 24%.

Development and destruction of the 680 pigment. The 680 absorbance in acetone appears immediately at the onset of oxidation and reaches a maximum when the 770 absorbance has dropped to about half its initial value. The rate at which the 680 pigment is subsequently destroyed by further oxidation depends markedly on the nature of the chromatophore sample. For example, a sample of rubrum was sonicated and centrifuged at 40,000 X g for 30 minutes (9), after which the supernatant (containing small chromatophore aggregates) and the residue (containing cellular debris and larger aggregates) were separately oxidized and extracted with acetone. With the smaller aggregates, the 680 absorption dropped off rapidly after reaching its maximum value, falling over 50% as the 770 absorbance (initial value 1.28) dropped from 0.75 to 0.30. With the larger aggregates, the 680 absorbance stayed near its maximum value until about 90% of the 770 pigment had disappeared. With both samples, however, the 680 absorbance in the region of maximum yield was about 45% of the initial absorbance at 770 mμ, indicating little difference in maximum yield in the two oxidations.

Separation procedure. Rubrum cells from 10 liters of culture (9, 4) were collected by centrifugation, buffered, sonicated, and recentrifuged at 40,000 X g for 25 minutes. The pigment could be obtained by oxidation either of the residue or the supernatant, but optimum oxidation conditions for the two fractions were generally different. Because of differences

among cell batches and variability of the content of oxidizable sulfur compounds in solution, a preliminary oxidation of a small portion of each batch was carried out to determine the optimum ratio of oxidant to 880 pigment. Typically, a 2.0 ml sample, optical density 10.5 at 880 mμ, (containing principally large chromatophore aggregates) required 1.6 mg of K_2IrCl_6 for the maximum yield of 680 pigment in the acetone extract.

The chromatophore suspension was cooled to 0° C, and the calculated quantity of K_2IrCl_6 solution (0.02 M) added dropwise with stirring. The preparation was allowed to stand for 10 minutes after addition of the oxidant, then added to 2.5 times its volume of acetone. The preparation was centrifuged, the supernatant taken, the residue extracted once with 75% acetone, and the extractions added to the supernatant. The combined solution was put onto a tightly-packed column of powdered polyethylene. (5) The column was first developed with 3/1 (v/v) acetone-water, which eluted both bacteriochlorophyll (blue) and an additional pigment (abs. max. approx. 730 mμ) which appeared to be formed from bacteriochlorophyll by air oxidation. Subsequent elution with 85/15 acetone-water separated the green pigment from the more strongly adsorbed pink carotenoids. Although the 680 pigment is much less sensitive to air oxidation than is bacteriochlorophyll, its solutions deteriorate slowly in air with the formation of a yellow pigment (which may be separated by rechromatographing on polyethylene); hence, all subsequent operations were carried out at or below room temperature. The solution of the green pigment in aqueous acetone was concentrated by bubbling dry nitrogen through rapidly. When a green oil separated, the preparation was extracted with chloroform (a small quantity of added sodium sulfate aids the separation of phases here), the chloroform extracts dried with anhydrous sodium sulfate, then

evaporated, again by passing dry nitrogen through. The highly colored (almost black) gum-like material which remained was very soluble in acetone, ether, the lower alcohols, chloroform, and carbon tetrachloride, giving brilliant green solutions in each. It could be precipitated from solution in ether or chloroform by addition of isopentane and cooling to -40°, but the "solid" became oily and sticky on being warmed to 0°. All attempts to crystallize the green pigment failed.

Spectra. The visible and near-ultraviolet spectrum of the pigment (approximately 10^{-4} molar in acetone) is shown in Figure 1, together with the product obtained by adding a trace of HCl.

The infrared spectrum ($DCCl_3$ solution), Figure 4, shows two bands (2800 and 2860 cm^{-1}) in the C-H stretching region and several in the C=O stretching region. The broad band at 3400 cm^{-1} might be hydrogen-bonded hydroxyl groups from the compound or from an impurity.

A satisfactory NMR spectrum of the pigment, either in deuteroacetone or deuterochloroform, was not obtained. Saturated (and very viscous) solutions of the pigment in $CDCl_3$ exhibited a number of proton resonances between 50 and 330 c.p.s. (Figure 5) relative to tetramethylsilane, possibly due in part to decomposition products. However, the resonances between 480 and 600 c.p.s. associated with the methine bridge protons in chlorophylls and in similar porphyrin derivatives (3) did not appear. No improvement in the spectrum could be achieved by diluting the solution or by transferring the pigment to deuteroacetone.

REFERENCES

1. A. F. H. Anderson and M. Calvin, Nature 194, 285 (1962).

2. R. K. Clayton, Photochem. Photobiol. 1, 203 (1962).

3. See, for example, G. L. Closs, J. J. Katz, F. C. Pennington, M. R. Thomas, and H. H. Strain, J. Am. Chem. Soc. 85, 3809 (1963); A. F. H. Anderson and M. Calvin, Arch. Biochem. Biophys. 107, 251 (1964).

4. C. Cohen-Bazire, W. R. Sistrom, and R. Y. Stanier, J. Cell. Comp. Physiol. 49, 25 (1957).

5. See, for example, K. E. Eimhjellen, O. Aasmundrud, and A. Jensen, Biochem. Biophys. Res. Comm. 10, 232 (1963).

6. Strong absorption at or near 680 mμ has been reported for a number of 2-acylchlorins and their magnesium derivatives. See, for example, O. T. G. Jones, Biochem. J. 91, 572 (1964); A. Stern and F. Pruckner, Z. physik. Chem. A 185, 140 (1939).

7. A. A. Krasnovskii and K. K. Voinovskaya, Doklady Akad. Nauk SSSR 81, 879 (1951).

8. I. D. Kuntz, Jr., P. A. Loach, and M. Calvin, Biophysical J. 4, 227 (1964).

9. P. A. Loach, G. M. Androes, A. F. Maksim, and M. Calvin, Photochem. Photobiol. 2, 443 (1963); R. H. Ruby, I. D. Kuntz, Jr., and M. Calvin, Proc. Nat. Acad. Sci. 51, 515 (1964).

10. W. M. Manning and H. H. Strain, J. Biol. Chem. 151, 1 (1943).

11. Commonly, although not invariably, the long wavelength absorption of a chlorophyll diminishes and the shorter wavelength absorption increases when conversion to the corresponding pheophytin occurs. See, for example, J. H. C. Smith and A. Benitez, Modern Methods of Plant Analysis, Vol. IV, Springer-Verlag, Berlin, 1955, p. 142.

FIGURES

Fig. 1. Spectra of the "680 pigment" and the product derived from it by acidification (about 10^{-4} M in acetone).

Fig. 2. Fall-off of photoinduced absorption changes in R. rubrum chromatophores with progressive bleaching of the absorption peak near 880 mμ.

Fig. 3. Decrease of absorbancies in R. rubrum chromatophores with progressive oxidation by Ir(IV), and decrease of absorbance at 770 mμ in acetone extracts derived from these chromatophores. (A) Absorbancies at 880 mμ. (B) Relative absorbancies at the (shifting) maximum near 880 mμ.

Fig. 4. Infrared spectrum of the "680 pigment" in $CDCl_3$.

Fig. 5. NMR spectrum of "680 pigment" in $CDCl_3$ (concentration unknown, but estimated at 0.02 M).

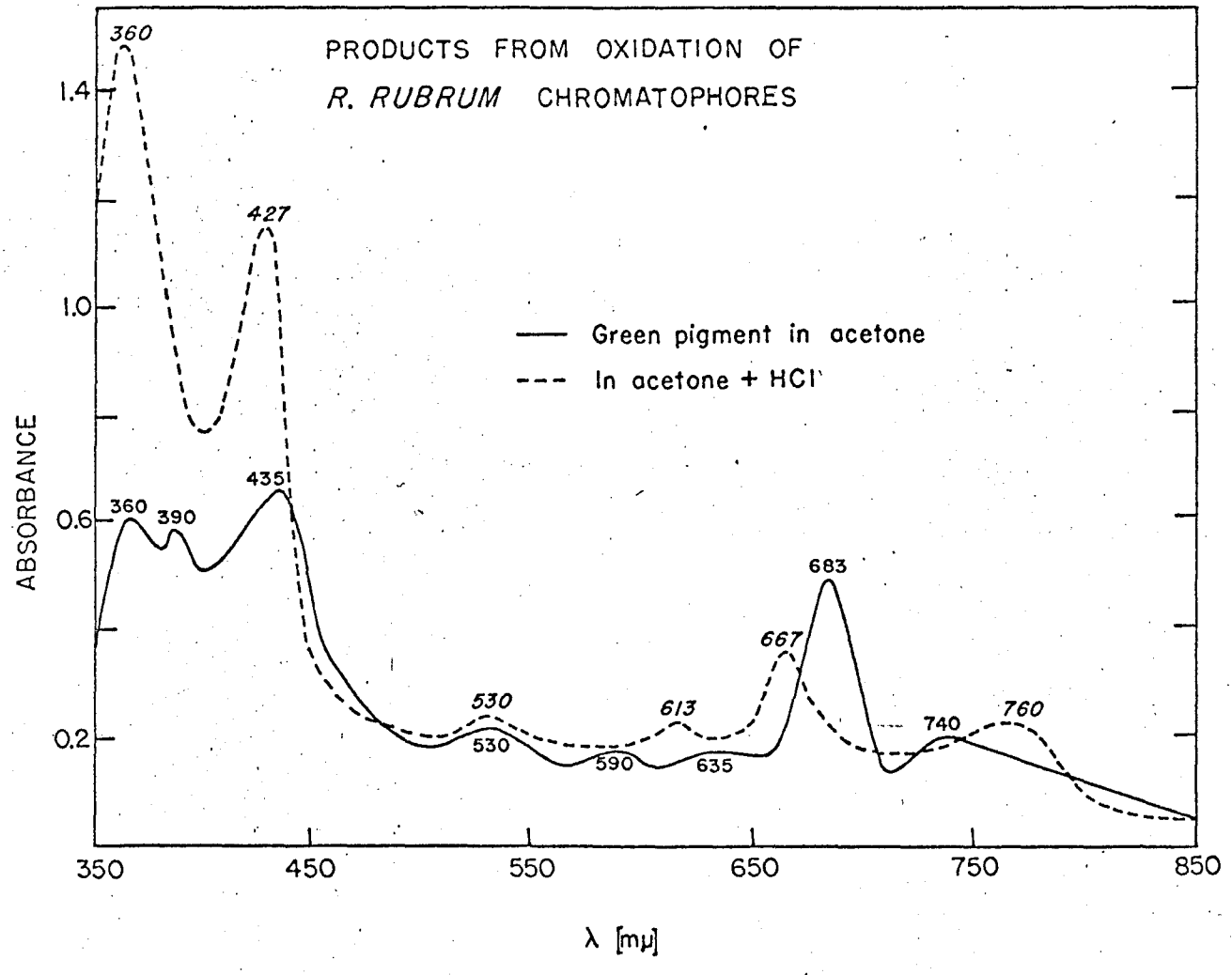

PRODUCTS FROM OXIDATION OF
R. RUBRUM CHROMATOPHORES
Green pigment in acetone
In acetone + HCl
ABSORBANCE
λ [mμ]
1.4
1.0
0.6
0.2
350
450
550
650
750
850
360
427
360
390
435
530
530
590
613
635
667
683
740
760
MUB-4104

Fig. 1.

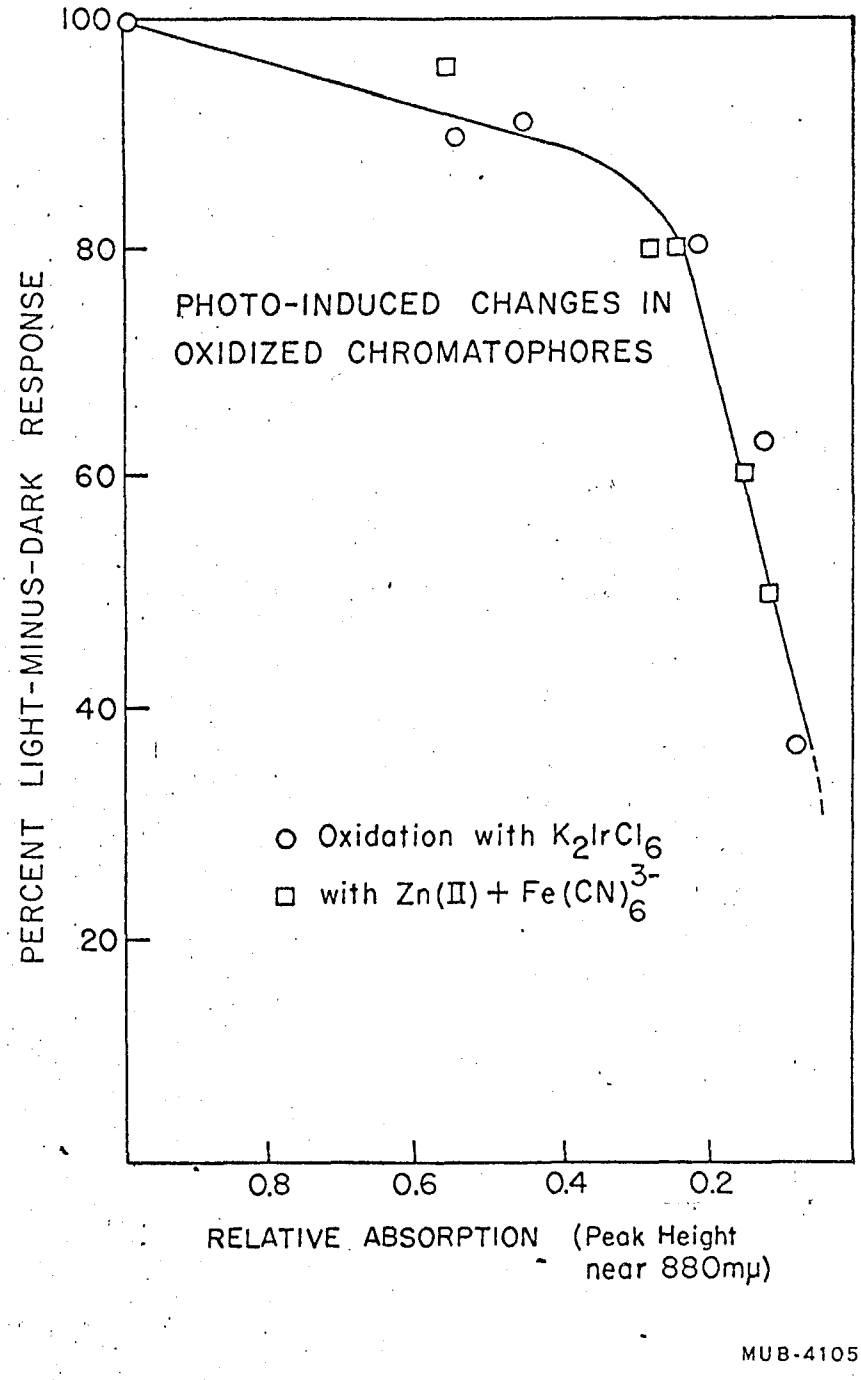

100
80
60
40
20
PERCENT LIGHT-MINUS-DARK RESPONSE
PHOTO-INDUCED CHANGES IN
OXIDIZED CHROMATOPHORES
O Oxidation with K_2IrCl_6
□ with $Zn(II) + Fe(CN)_6^{3-}$
0.8
0.6
0.4
0.2
RELATIVE ABSORPTION (Peak Height near 880mμ)
MUB-4105

Fig. 2.

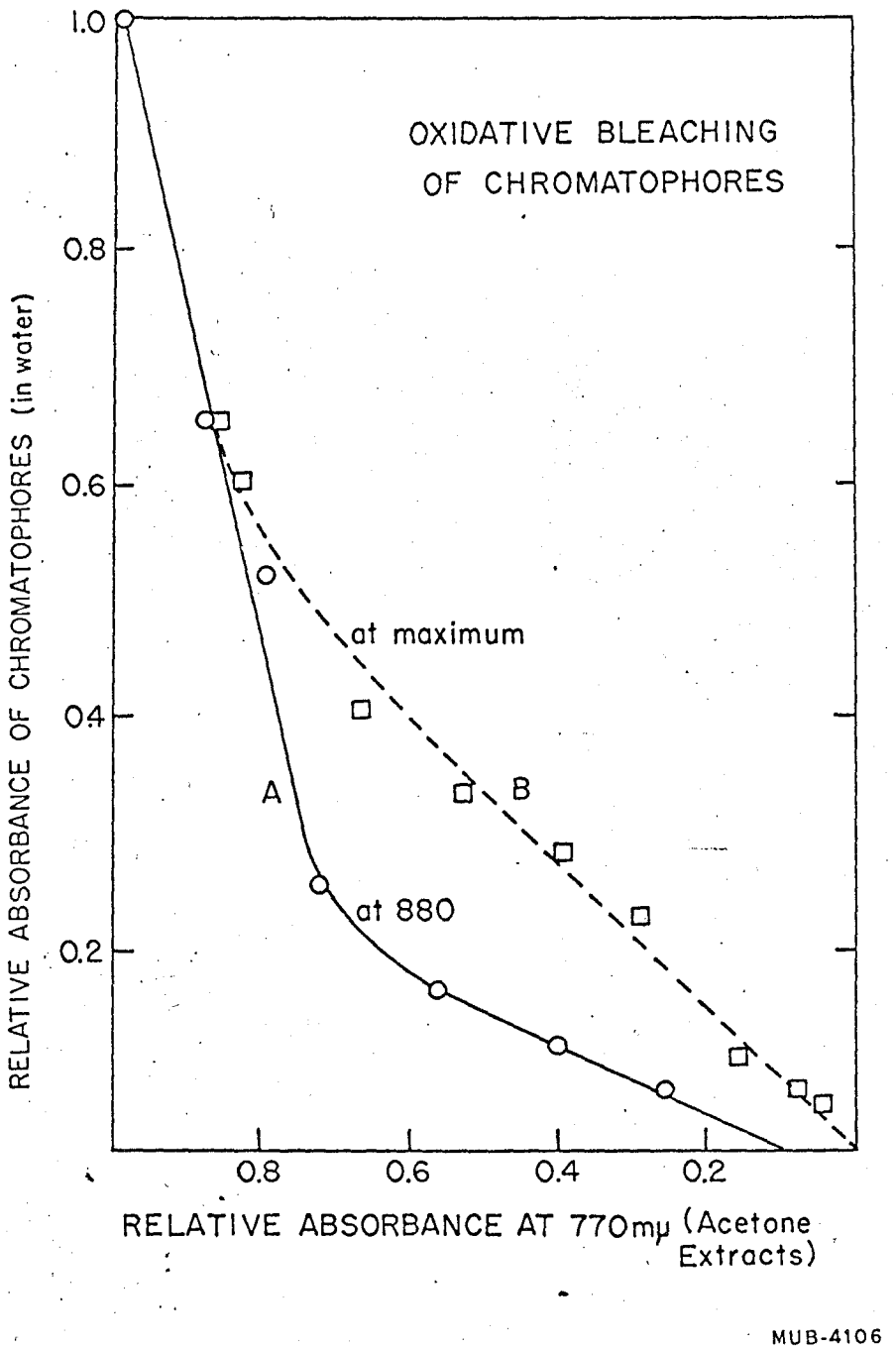
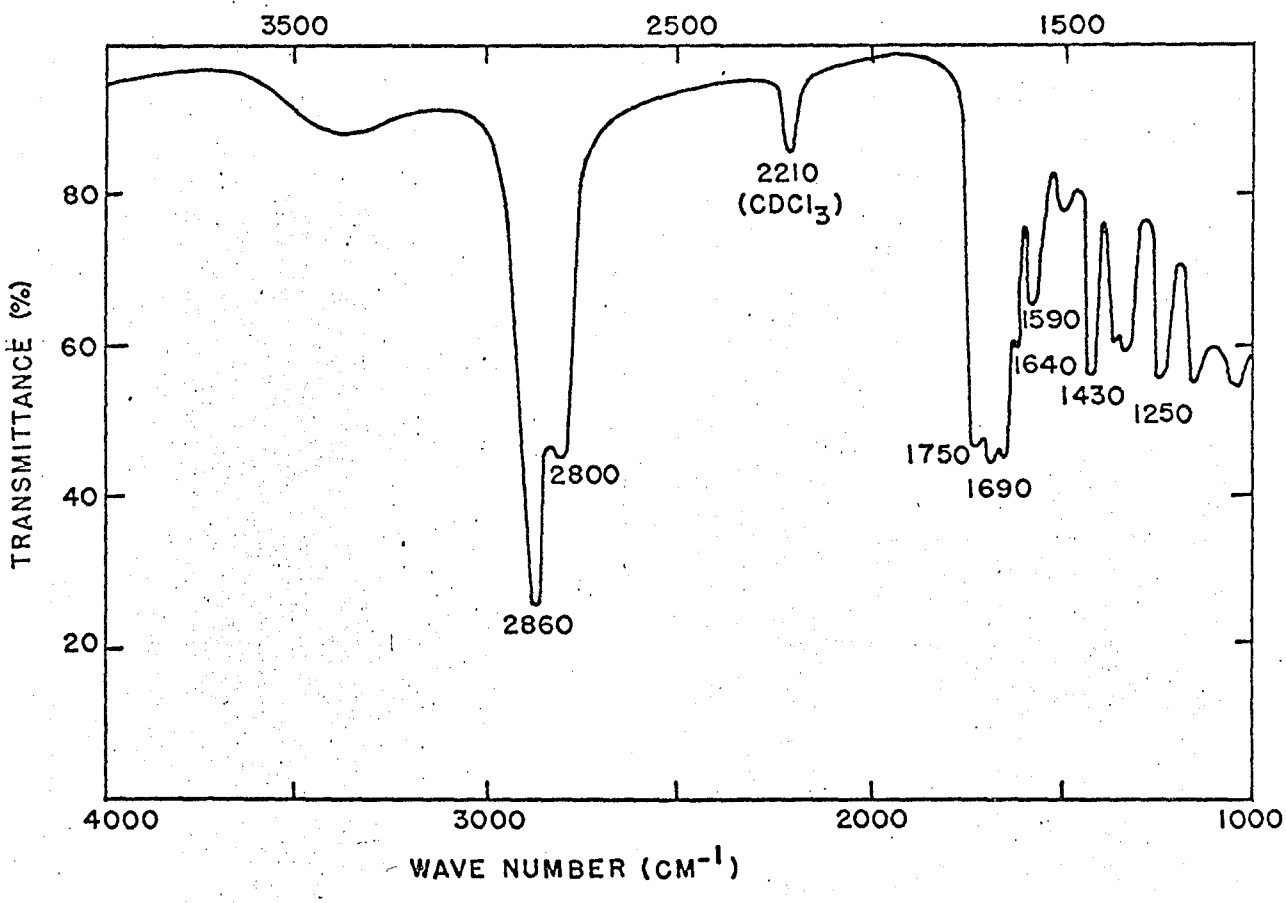

OXIDATIVE BLEACHING
OF CHROMATOPHORES
RELATIVE ABSORBANCE OF CHROMATOPHORES (in water)
1.0
0.8
0.6
0.4
0.2
at maximum
A
B
at 880
0.8
0.6
0.4
0.2
RELATIVE ABSORBANCE AT 770mμ (Acetone Extracts)
MUB-4106

Fig. 3.



MUB-4107

Fig. 4.

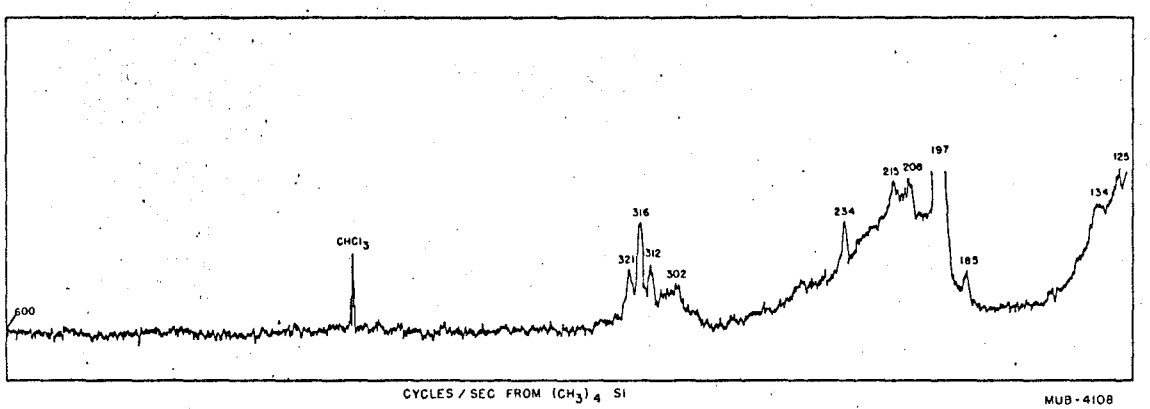

600
CHCl3
316
321
312
302
234
215
206
197
185
134
125
CYCLES / SEC FROM (CH3)4 Si
MUB-4108

Fig. 5.